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AGRICULTURE DEVELOPMENT FUND

FINAL REPORT

20010306

DEVELOPMENT OF NEEDLE FREE VACCINE DELIVERY SYSTEMS FOR CATTLE

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FINAL REPORT

AGRICULTURE DEVELOPMENT FUND

PROJECT # 20010306

"Development of needle free vaccine delivery systems for cattle"

By

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A) ABSTRACT

The main objective of the work was to construct and characterize recombinant BAdV-3 expressing three vaccine antigens of different pathogens and evaluate its potential in inducing immune responses in animals. We demonstrated the feasibility of constructing recombinant BAdV-3 expressing three viral antigens. We also demonstrated the potential of the recombinant BAdV-3 expressing three viral glycoproteins in inducing immune responses. However, insertion of three foreign genes in the genome of BAdV-3 drastically reduces the growth of recombinant BAdV-3 thus making it difficult to produce recombinant virus economically.

B) EXECUTIVE SUMMARY

Vaccination against infectious diseases is considered to be one of the major advances in modern medicine when one considers the reduction in human and animal suffering. Sub-unit vaccines often do not induce breadth of immunity required (e.g. cell mediated immunity etc.), and they require strong adjuvants, of which few are licensed. Furthermore, producing sub unit vaccines for use in veterinary medicine, using modern biotechnology approaches is dictated by economics. Thus, novel approaches are required to produce animal vaccines to reduce animal suffering and productivity. One feasible way of achieving this is to develop safe, effective and economically viable live-vectored vaccines. Ourmain aim is to develop bovine adenovirus -3 (BAdV-3) based live viral vector. The BAdV-3 infects calves but often does not produce disease. Earlier, we characterized BAdV-3 at the molecular level by determining the complete genome sequence

and transcriptional map. We have identified different essential and non essential regions / sites of BAdV-3 genome, which can be deleted and used for insertion of foreign genes. We have also demonstrated the feasibility of constructing BAdV-3s expressing single or two vaccine antigens. Here, we demonstrate the feasibility of constructing and using recombinant BAdV-3 (expressing three vaccine antigens) in inducing immune responses. Although viable recombinant BAdV-3 expressing three vaccine antigens could be isolated, the recombinant BAdV-3 replicates poorly. However, further experiments need to be done before full potential of this approach (BAdV-3 vector expressing three vaccine antigens) can be realized.

C) TECHNICAL REPORT

i) Background

The use of vaccines is an effective method of stimulating the naive animal to develop protective immunity against the disease causing organism. Since many disease causing organisms enter at mucosal surfaces (respiratory and gastrointestinal tract), immunity is required at the mucosal site to block the initial infection and thus reduce the chances of development of disease. This can be best achieved by direct infection of respiratory or gastrointestinal tract. Many of the available vaccines are delivered by **intramuscular injection**, which does not induce mucosal immunity but results in the development of **injection site reactions**, which adversely **affect the meat quality**. These vaccines require strong oil based **adjuvants** of which very few are licenced for veterinary use. In many cases, vaccination has to be **repeated annually** due to limited duration of

immunity. One way to produce safe and cost effective vaccines is to develop live vectored vaccines. Such live vectors can be engineered to carry genes for other pathogens thus making it possible to immunize animals to produce protective immunity at the mucosal surface to various disease organisms at one time. We have chosen to develop bovine adenovirus-3 based viral vectored vaccines. Earlier studies from my laboratory have established **the safety and efficacy** of the bovine adenovirus vector for use as an intranasal vaccine delivery system in cattle. These studies have provided **the foundation for establishing a family of safe and effective vaccines against respiratory and enteric disease(s) of cattle using bovine adenovirus as a vector.** To express more than one vaccine antigens, recently, we have identified two additional regions (E1 and E4), which could be utilized for the insertion of foreign genes. Since E1 region is essential for virus replication, E1 deleted BAdV-3 could only grow in a cell line expressing E1 proteins. We have now established two cell lines expressing E1 proteins, constructed E1 deleted BAdV-3 and showed that virus is **avirulent** in cattle. In addition, we have also constructed E4 deleted BAdV-3. By replacing these deleted genes with the protective antigens of other respiratory pathogens of cattle, **we can make single shot multivalent vaccine, which when delivered intranasally, can induce immunity to a variety of different important pathogens of cattle.** *We propose to construct and characterize recombinant **BAdV-3 expressing three vaccine antigens of different viral pathogens,** and evaluate their potential in inducing protective immune responses in cattle. This will not **only improve vaccination**, but will also **reduce the cost of vaccines for livestock producers**.*

ii) Objectives

The main objective is to construct and evaluate the efficacy of recombinant bovine adenovirus-3 expressing three vaccine antigens of different viral pathogens.

The specific aims are:

a) Construction of plasmids containing gD (BHV-1), gG (BRSV), gp53 (BVDV) and/or HE (BCV).

b) Construction of full length BAdV-3 genomic clones containing different vaccine antigens

c) Construction and characterization of recombinant BAdV-3 expressing vaccine antigens

d) Test the efficacy in inducing immune responses.

iii) Results and discussion

Previously, we have developed an efficient and reliable method of isolating recombinant BAdV-3 by constructing an infectious full length BAdV-3 genomic clone in a plasmid using homologous recombination machinery of *E. coli* (Fig. 1). Using a similar approach, we also constructed a) an E3 deleted BAdV-3 genomic DNA clone which when transfected in bovine cells produced virus named BAV302 (Fig. 2), b) an E3 –E4 deleted BAdV-3 genomic DNA clone which when transfected into bovine cells produced virus named BAV429 (Fig. 3) and c) an E1A-E3 deleted BAdV-3 genomic DNA clone which when transfected into VIDO R2 (E1 complementing) cells produced virus named BAV500 (Fig. 4).

In order to construct recombinant BAdV-3 expressing three genes, first we attempted to use the strategy of fusing the coding sequences of three genes in such away that the coding sequences are separated by IRES sequences from different sources. This will produce a single mRNA in which the 5' gene will be translated by ribosomal scanning method while the 3' gene will be translated by internal ribosomal entry site mechanisms.

a) Insertion of multiple genes in E3 region

Construction of plasmid pFBAV1002

Initially, we constructed a plasmid (pCite.G) in which we fused bovine respiratory syncytial virus (BRSV) glycoprotein G coding sequence, 3' to IRES sequence of endomyocarditis virus. A 1.3 kb DNA fragment (containing bovine herpesvirus-1 (BHV-1) glycoprotein gDt) of pRSVgDt was isolated and ligated to EcoRI digested pCite.G creating plasmid pDemcG. A 25 bp oligonucleotide containing IRES sequences of GtX protein was fused in frame to bovine viral diarrhea virus (BVDV) gp53 coding sequence in a plasmid pGP53a creating plasmid pGP53.gtx. A 1.1 kb DNA fragment was isolated from pGP53.gtx (containing gIRES-gp53) and ligated to EcoRV digested pDemcG creating plasmid pgDt.G.gp53. Finally, 3.9 kb fragment (gDt-IRES-G-gIRES-gp53) was isolated from pgDt.G.gp53 and ligated to SrfI digested pBAV301 creating plasmid pBAV301.DG53. The schematic diagrams of the plasmids are given in fig 5.

Using homologous recombination machinery of *E. coli*, a 7.9kb fragment isolated from plasmid pBAV301.DG53 was recombined with SrfI digested pFBAV429 in *E. coli*

BJ5183 to create plasmid pFBAV1002 (Fig. 6). In order to isolate a recombinant virus, Madin Darby bovine cells or VIDO R2 cells (fetal bovine retina cells transformed by E1 region of human adenovirus type 5) were transfected with PacI digested **pFBAV1002** plasmid DNA and observed for the development of cytopathic effects. Repeated transfections of bovine cells did not produce any recombinant virus.

b) Insertion of multiple genes in E3-E4 region

Construction of plasmid pFBAV1004

Initially, a 2.9 kb fragment was isolated from plasmid pDemcG and ligated to SrfI digested pBAV301 creating plasmid pBAV301DemcG. A 8.1 kb fragment was isolated from pBAV301DemcG and recombined with SrfI digested pFBAV429 in *E. coli BJ 5183* to create plasmid pFBAV1003 (Fig. 7).

Next, we constructed an E4 transfer vector pBAV429 by ligating 3.7kb ScaI – HpaI fragment isolated from plasmid E4polyIISyn with SrfI linker. A 1.1 kb DNA fragment containing gp53 gene was ligated to HindIII digested pcDNA3 to create plasmid pcDNA.gp53. A 1.6 kb fragment containing gp53 flanked by CMV IE promoter and BGH polyA signal sequences was isolated from plasmid pcDNA.gp53 and ligated to SrfI digested pBAV429 to create plasmid pE4.gp53 (Fig.7). Finally, a 4.5 kb BamHI fragment was isolated from plasmid pE4.gp53 and recombined with SwaI digested pFBAV1003 to create **pFBAV1004** (Fig. 8). The identity of the plasmid pFBAV1004 was confirmed by restriction enzyme analysis of plasmid DNA.

After performing several transfections, the cytopathic effects were visible in few cells. After successive passages, 70%-80% of the cells showing cytopathic effects were collected, freeze-thawed and the recombinant BAV1004 was propagated. Analysis of the replication of recombinant virus suggests that the BAV1004 grows slowly. Restriction enzyme (RE) analysis suggested that RE pattern of passage 1 virus was correct, However, the RE pattern of passage 10 was aberrant suggesting that recombinant virus genome was not stable.

Construction of pFBAV1006

Initially, a 1.1 kb DNA fragment containing BVDV gp53 gene was isolated from pSin-E2 and ligated to EcoRI digested pCite.G to create plasmid pgp53.G. A 2.7 kb DNA fragment (containing gp53-IRES-G) was isolated from plasmid pgp53-IRES-G and ligated to SrfI digested pBAV301 to create plasmid pgp53emcG. Finally, a 8.1 kb DNA fragment isolated from plasmid pgp53emcG was recombined with SrfI digested pFBAV429 in *E. coli BJ5183* to create plasmid pFBAV1005 (Fig. 9).

Next, a 1.3 kb DNA fragment containing BHV-1 gDt gene was ligated to HindIII digested pcDNA3 to create plasmid pcDNA3,gDt. A 2.0 kb fragment containing gDt flanked by CMV IE promoter and BGH polyA signal sequences was isolated from plasmid pcDNA.gDt and ligated to SrfI digested pBAV429 to create plasmid pE4.gDt (Fig. 9). A 5 kb BamHI fragment was isolated from pE4.gDt and recombined with SwaI digested pFBAV1005 to create **pFBAV1006** (Fig. 10).

The identity of the pFBAV1006 was confirmed by restriction enzyme analysis of plasmid DNA. The PacI digested plasmid DNA was transfected into bovine cells. The

transfected cells showing cytopathic effects were collected, freeze-thawed and used for isolation of viral DNA. The restriction enzyme analysis of the viral DNA confirmed the identity of the recombinant BAV1006 (Fig. 11). Analysis of the replication of recombinant virus suggests that BAV1006 grows very slowly. Surprisingly, analysis of RE pattern of viral DNA suggested that the genome of this recombinant virus was also not stable.

Construction of plasmid pFBAV1007

Initially, a 1.3 kb DNA fragment containing BCV HE gene was ligated to HindIII digested pcDNA3 to create plasmid pcDNA3.HE (Fig. 12). A 1.9 kb fragment containing HE flanked by CMV IE promoter and BGH polyA signal sequence was isolated from plasmid pcDNA3.HE and ligated to SrfI digested pBAV429 to create plasmid pE4.HE (Fig. 12).The identity of pE4.HE was confirmed by restriction enzyme analysis of plasmid DNA.

Earlier, we also constructed pFBAV1003 (Fig. 7) containing gDt + gG inserted in E3 region of plasmid pFBAV429. In order to construct pFBAV1007 (Fig. 13), 5.1kb fragment (containing BCV HE gene) was isolated from plasmid pE4.HE and recombined (using *E. coli* BJ5183) with SwaI digested pFBAV1003 (Fig. 7). The identity of pFBAV1007 (Fig. 13) was confirmed by restriction enzyme analysis of plasmid DNA . The PacI digested pFBAV1007 DNA was transfected into bovine cells. After several transfections and repeated passaging of transfected cells, cytopathic effects were visible in few transfected cells. The virus was expanded and the identity of recombinant virus was confirmed by restriction enzyme analysis of virion DNA (Fig. 14). Moreover,

recombinant virus genome appeared to be stable after 10 passages. However, the recombinant virus titers were 2 to 2.5 logs less than the BAdV302 (control) virus.

d) Test the efficacy in inducing immune responses

In order to determine the ability of recombinant BAdV3 (expressing three vaccine antigens) to induce immune responses, we immunized animals with recombinant BAV1007. Three to four week old calves were immunized twice, 4 weeks apart with 10^6 PFU of BAV1007 or BAV302 (control) recombinants. Serum samples were collected at different times post immunization and analyzed by ELISA. Low levels of BHV-1 and BCV specific IgG antibodies could be detected in animals vaccinated with BAV1007 (Fig. 15). However, no BRSV specific antibodies could be detected in animals vaccinated with BAV1007. The animals immunized with BAV302 did not show any BHV-1/BRSV/BCV IgG antibody. Nasal secretions collected at different times post immunization were analyzed for BRSV/BHV-1/BCV specific IgA antibodies by ELISA. No significant titer of virus specific IgA was found in calves immunized with BAV1007. Similarly, no significant virus (BHV-1/BRSV/BCV) neutralizing antibodies were detected in calves immunized with recombinant BAV1007.

iv) Conclusion

One of the major impediments to effective vaccines is delivery. We have choosen to develop BAdV-3 as a live viral vector for delivery of vaccine antigens. Initially, we developed a simple and efficient way of constructing recombinant BAdV-3 (using homologous recombination machinery of *E. coli).* Next, we identified different

regions of the BAdV-3 genome (E1, E3, E4), which can be deleted and thus used for the insertion of vaccine antigen genes. This has increased the insertion capacity of replication competent BAdV-3. In addition, we have demonstrated the construction and characterization of recombinant BAdV-3 vectors expressing single or two vaccine antigens.

Although we could demonstrate the feasibility of constructing recombinant BAdV-3 expressing three antigens, recombinant BAdV-3 expressing three antigens grew less efficiently than control BAdV-3. Moreover, the stability of the recombinant genome(s) depended on the nature of foreign gene insertion. Immunization of calves with recombinant BAdV-3 expressing three antigens produced low levels of virus specific IgG antibodies. More studies need to be done to determine the full potential of this approach for delivering vaccines in porcine.

v) Information of benefit to producers, processors or government

Significant economic losses (mortality, loss of productivity, treatment costs etc.) in the Saskatchewan cattle industry are caused by infection (respiratory diseases $ 1 billion and enteric diseases $300 millions in North America). These losses could be prevented by developing effective preventive measures such as vaccination. This proposal describes a unique approach to the production of a new generation of safe, cost effective and highly efficacious vaccines for animals. Calves at birth can be vaccinated by intranasal administration of recombinant bovine adenovirus thus circumventing the problem of passive immunity the calf receives from the dam. In addition to providing long duration immunity, this route of immunization will induce mucosal immunity at the

gastrointestinal tract due to common mucosal immune response. In addition, intranasal immunization does not cause damage to meat quality and does not need adjuvant. Finally, effective disease prevention by vaccination will reduce the level of antibiotic residues in the meat.

D) PERSONNEL

Dr. Suresh K. Tikoo	Senior Research Scientist
Dr. Xing Li	PDF
Dr. Alexander Zakhartchouk	Research Scientist
Dr. G. Mutwiri	Research Scientist
Ms Kyla/Jill/Betty	Technician(s)

E) EQUIPMENT

i) Microfuge centrifuge	$4329.98
ii) Protein gel apparatus	$3349.37

F) PROJECT DEVELOPED MATERIAL

So far we have not published any material yet.

G) PROJECT DEVELOPED PHOTOS

Not applicable

H) ACKNOWLEDGEMENT

I would like to thank Drs. X. Li, Alexander Zakhartchouk, and Yan Zhou for their participation in this project as well as Kyla Shea for excellent technical assistance. I would also like to thank Dr. Don Wilson, Mr. Barry Carroll and the rest of VIDO animal care staff for their willing assistance and advice on all work involving animals. In

addition, Drs. George Mutwiri (immunology) and Phil Wilson (animal challenge) provided excellent support and advice.

Finally, we thank Saskatchewan Agriculture Development Fund for their financial support of this project. We will acknowledge the support of the department when we publish these results.

BAV-3 FULL LENGTH GENOMIC CLONE IS INFECTIOUS

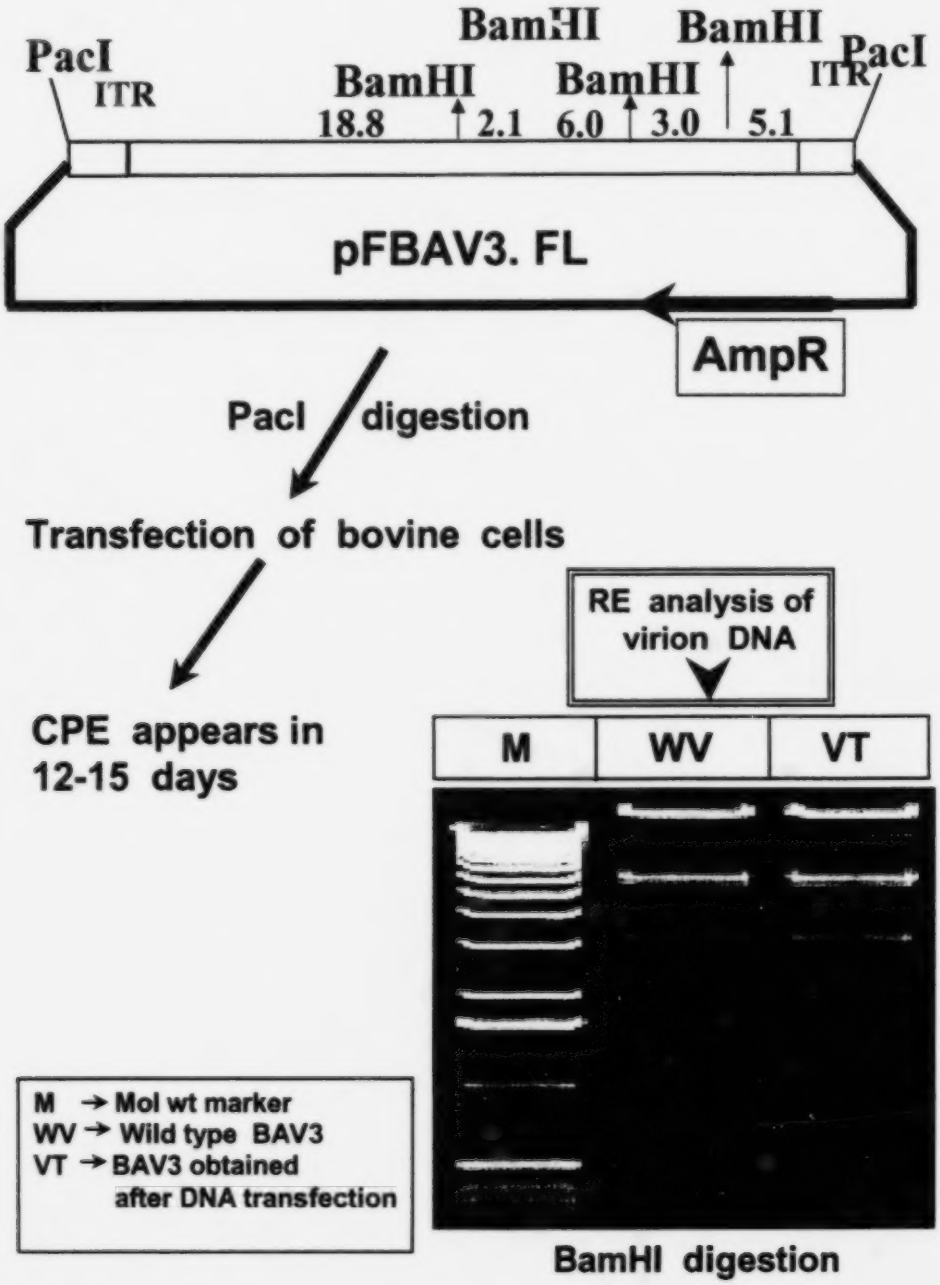


FIG. 1

CONSTRUCTION OF E3 DELETED BAV-3 (BAV302)

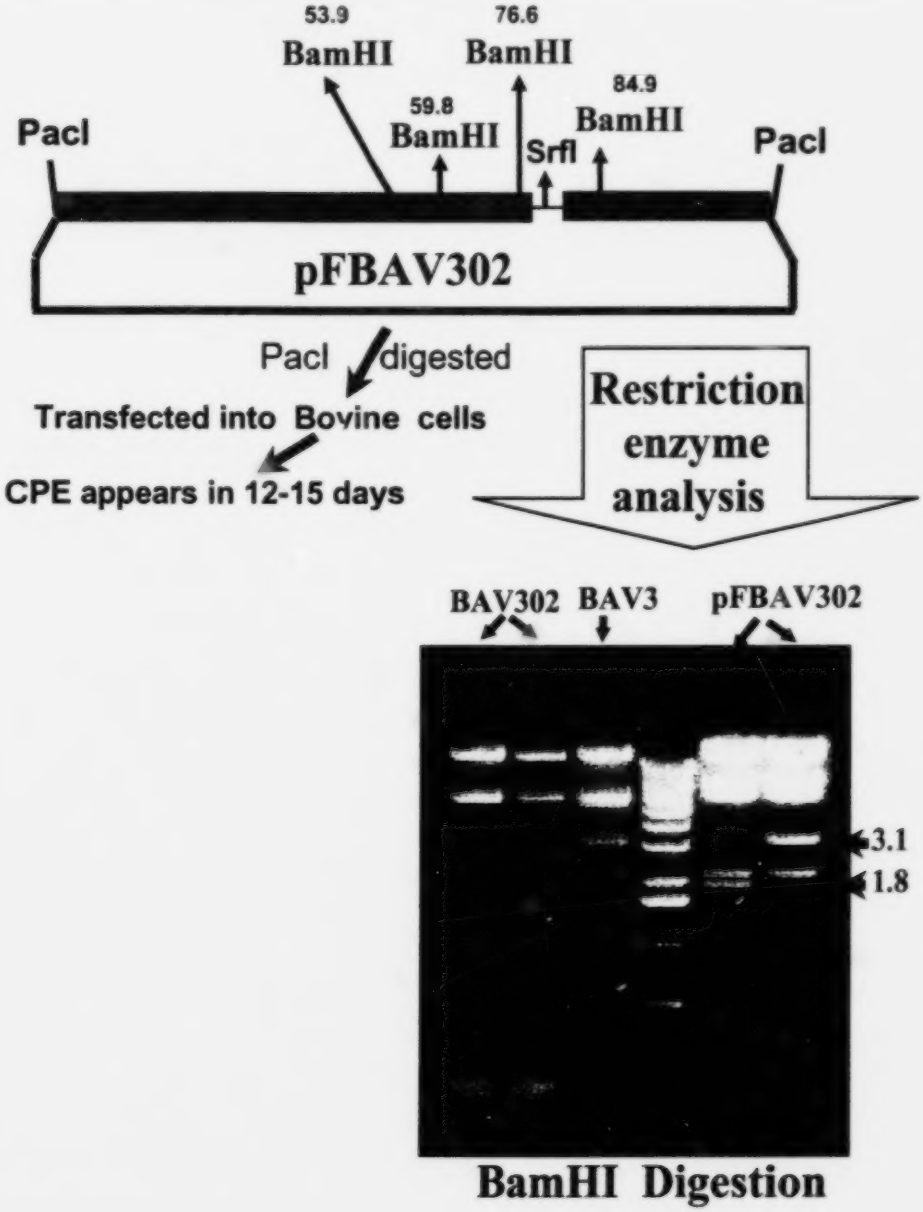


FIG. 2

CONSTRUCTION OF RECOMBINANT BAV429 AND BAV430

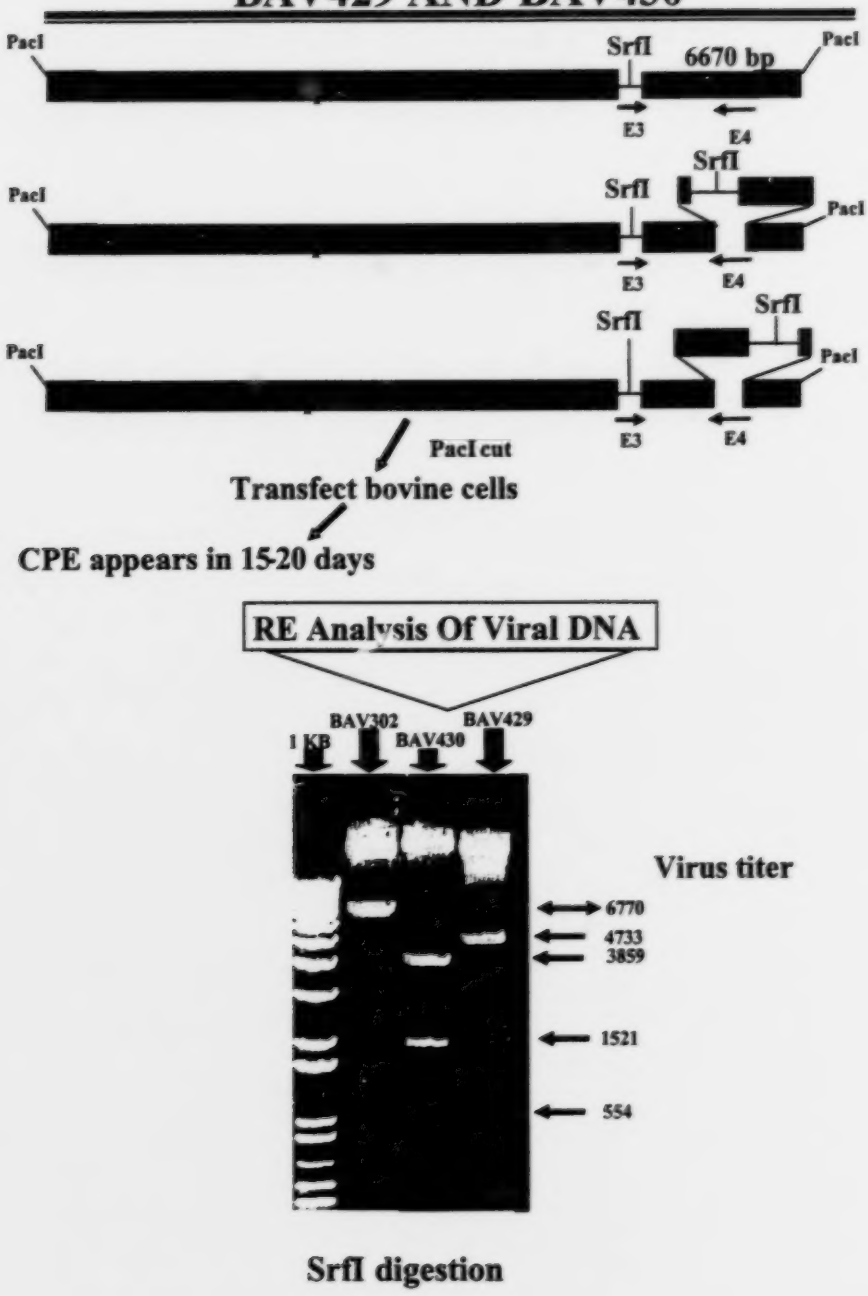
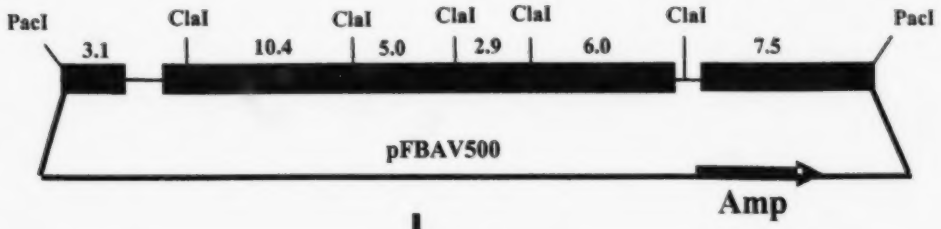


FIG. 3

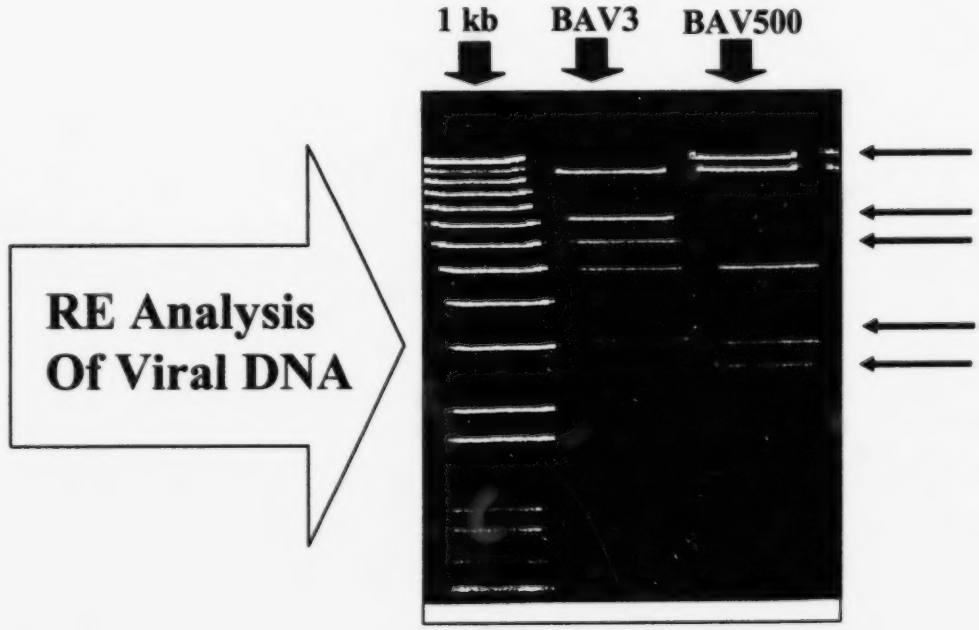
CONSTRUCTION OF E1A-E3 DELETED BAV-3



PacI cut

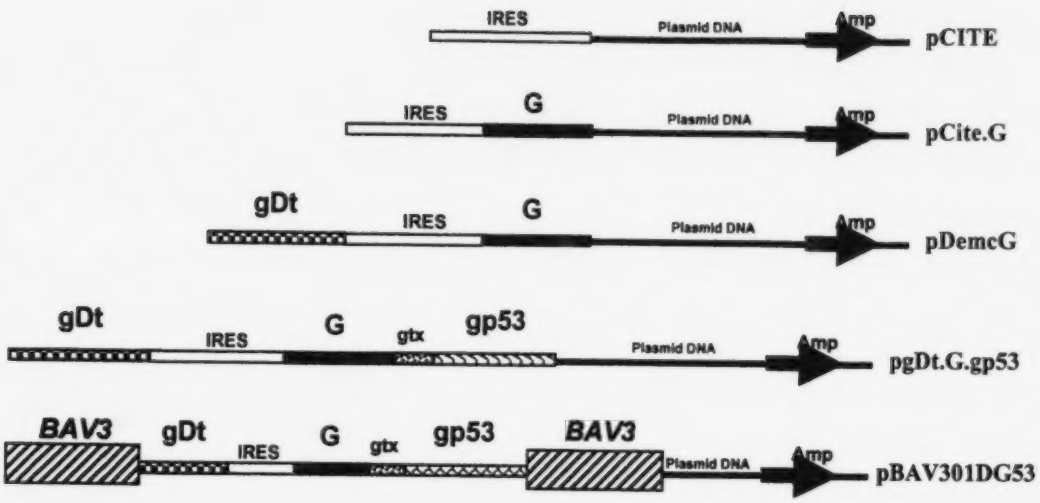
Transfect VIDO R2 cells

CPE appears in 10-20 days (BAV500)



ClaI digestion

FIG. 4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Amp
pCITE
IRES
G
Plasmid DNA
Amp
pCite.G
gDt
IRES
G
Plasmid DNA
Amp
pDemcG
gDt
IRES
G
gtx
gp53
Plasmid DNA
Amp
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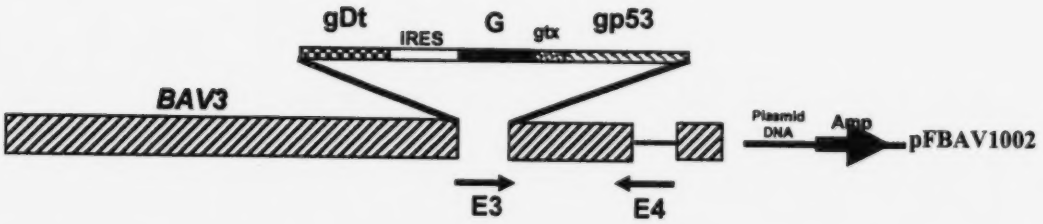
Amp
pBAV301DG53

FIG. 5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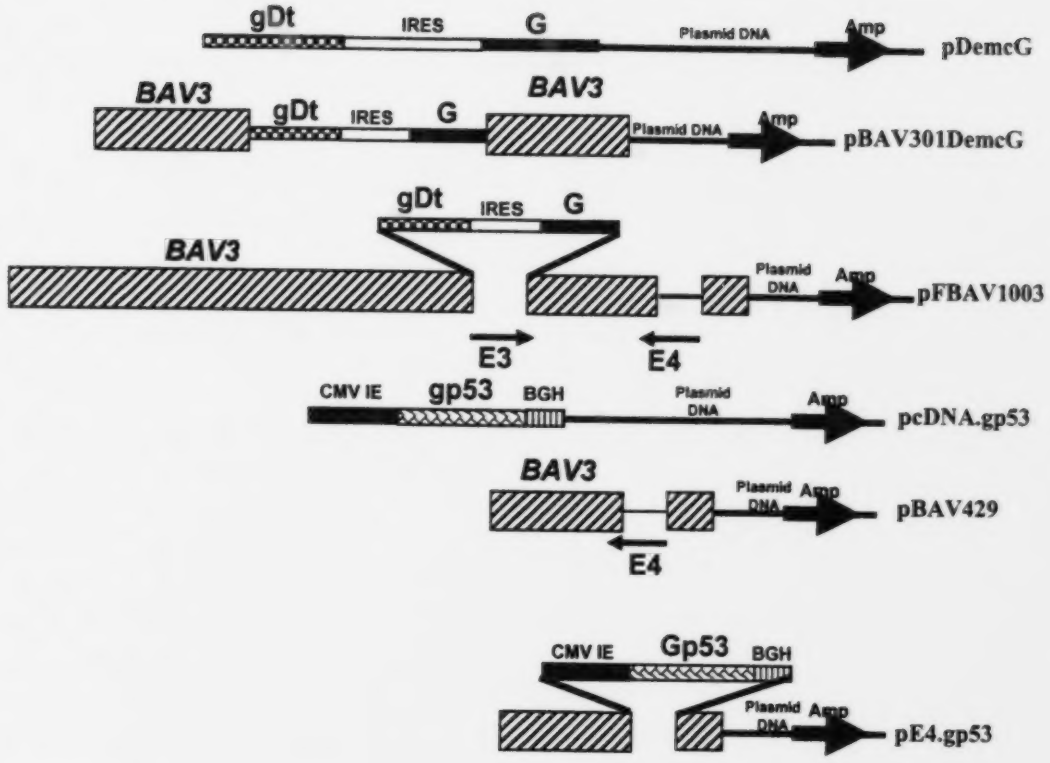
E3
E4

FIG. 6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gDt
IRES
G
BAV3
Plasmid DNA
Amp
pFBAV1003
E3
E4
CMV IE
gp53
BGH
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pcDNA.gp53
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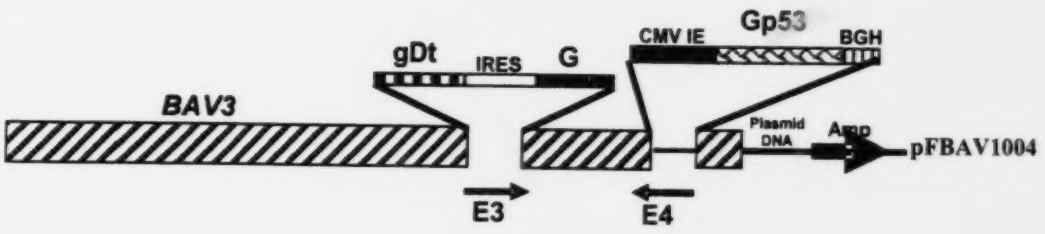
pE4.gp53

Fig. 7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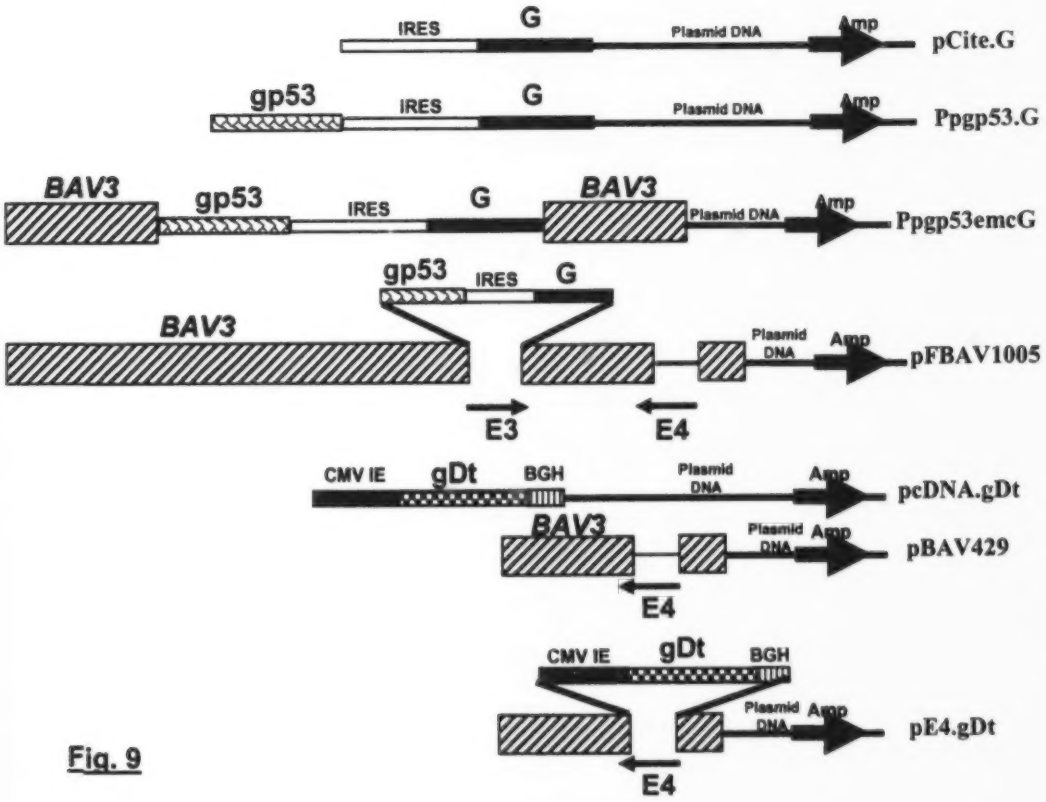
Plasmid DNA
Amp
pFBAV1004
E3
E4

Fig. 8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pFBAV1005
E3
E4
CMV IE
gDt
BGH
Plasmid DNA
Amp
pcDNA.gDt
BAV3
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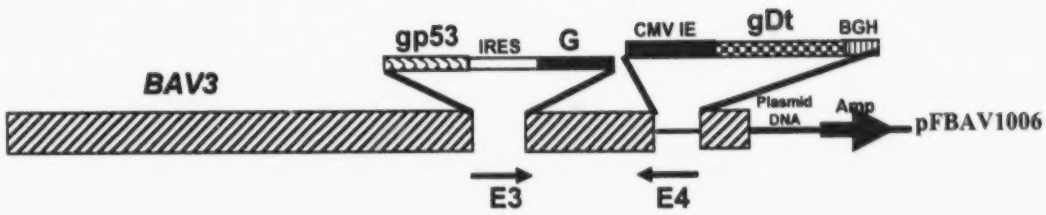
E4

Fig. 9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E4

Fig. 10

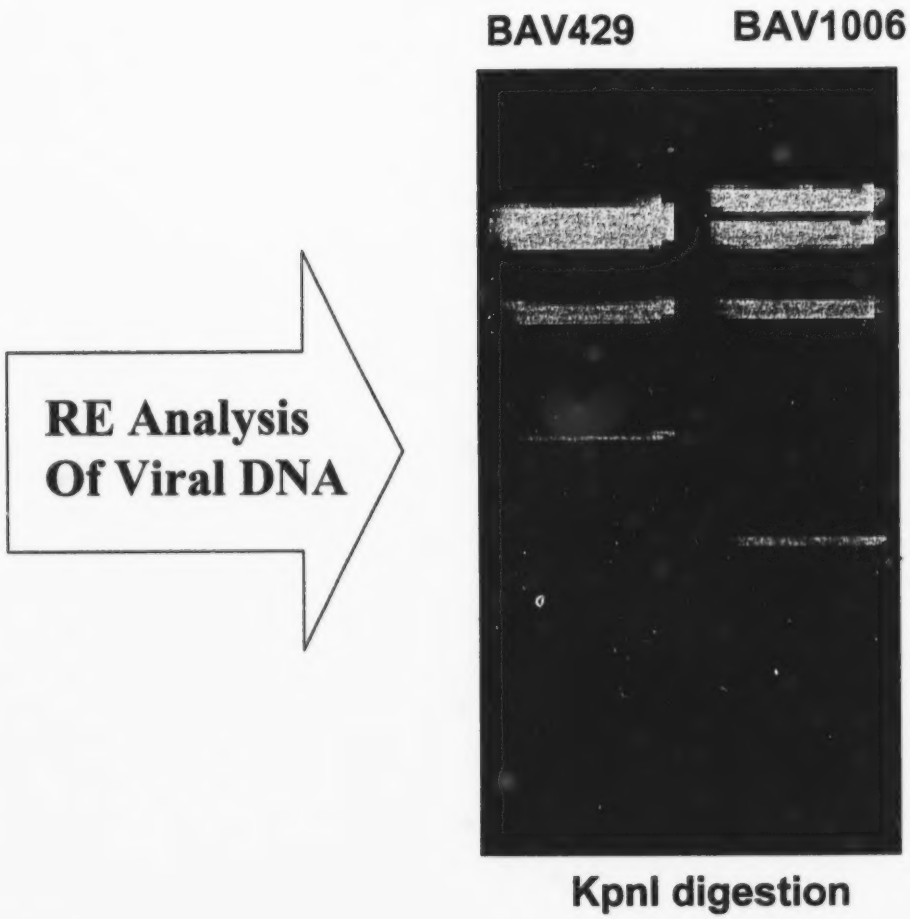
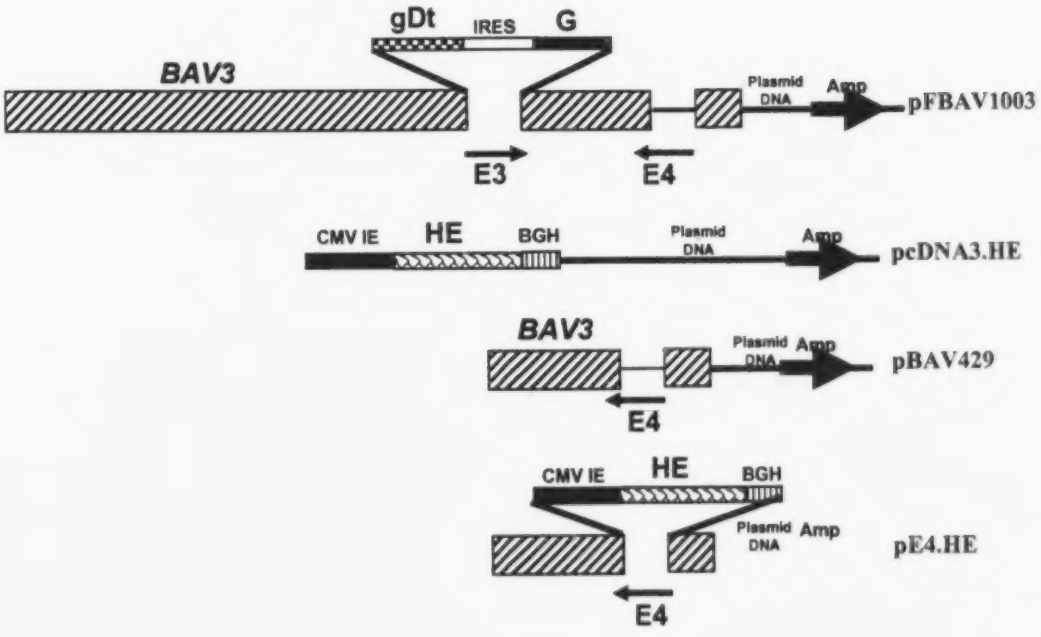


Fig. 11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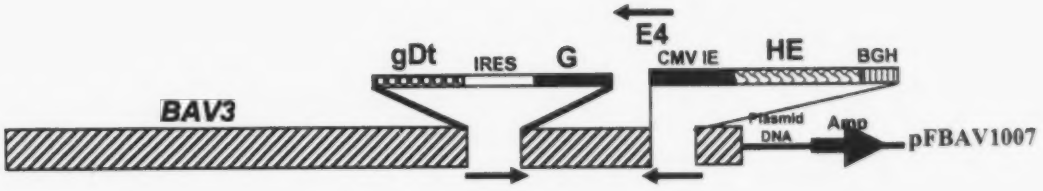
E4

Fig. 12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CMV IE
HE
BGH
BAV3
Plasmid
DNA
Amp
pFBAV1007

Fig. 13

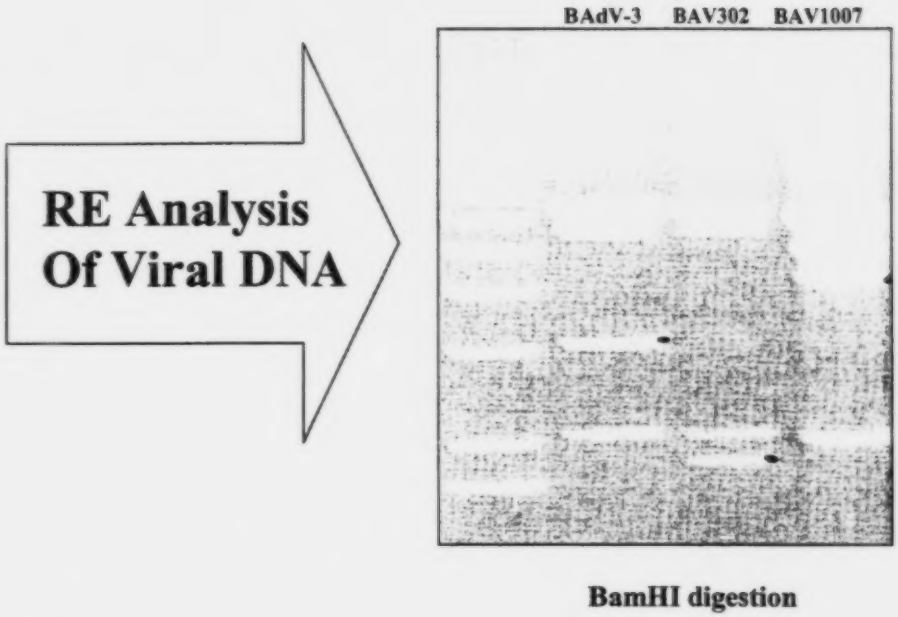
RE Analysis
Of Viral DNA
BAdV-3
BAV302
BAV1007
BamHI digestion

Fig. 14

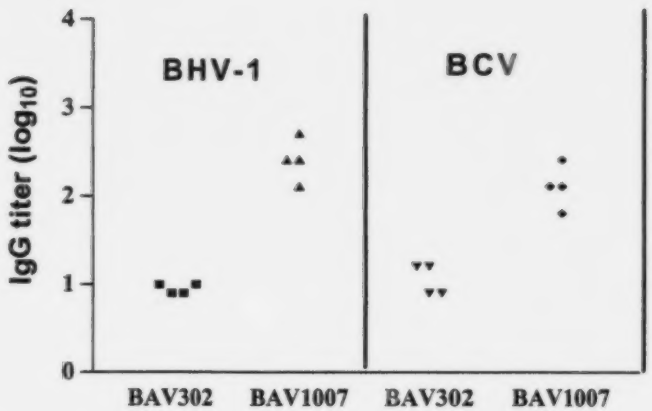
IgG titer (log10)
4
3
2
1
0
BHV-1
BCV
BAV302
BAV1007
BAV302
BAV1007

Fig. 15